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INSTITUTE REPORT NO. 123

CORNEAL ENDOTHELIUM AFTER INFRARED LASER EXPOSURE
Evaluation of Techniques to Study Corneal Lesions

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**Corneal Endothelium after Infrared Laser Exposure
Evaluation of Techniques to Study Corneal Lesions - Schuschereba et al.**

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REPORT DOCUMENTATION PAGE		READ INSTRUCTIONS BEFORE COMPLETING FORM
1. REPORT NUMBER LAIR Institute Report No. 123	2. GOVT ACCESSION NO. AD-A116	3. RECIPIENT'S CATALOG NUMBER 748
4. TITLE (and Subtitle) Corneal Endothelium After Infrared Laser Exposure. Evaluation of Techniques to Study Corneal Lesions		5. TYPE OF REPORT & PERIOD COVERED Final 1975-1982
		6. PERFORMING ORG. REPORT NUMBER
7. AUTHOR(s) S. Schuschereba, MA B. Stuck, MS P. Shawaluk, MD		8. CONTRACT OR GRANT NUMBER(s)
9. PERFORMING ORGANIZATION NAME AND ADDRESS Divisions of Ocular Hazards & Research Support, Letterman Army Institute of Research, Presidio of San Francisco, CA 94129		10. PROGRAM ELEMENT, PROJECT, TASK AREA & WORK UNIT NUMBERS
11. CONTROLLING OFFICE NAME AND ADDRESS U.S. Army Medical Research & Development Command Fort Detrick, Frederick, MD 21701		12. REPORT DATE June 1982
		13. NUMBER OF PAGES 17
14. MONITORING AGENCY NAME & ADDRESS (if different from Controlling Office)		15. SECURITY CLASS. (of this report) UNCLASSIFIED
		15a. DECLASSIFICATION/DOWNGRADING SCHEDULE
16. DISTRIBUTION STATEMENT (of this Report) APPROVED FOR PUBLIC RELEASE; DISTRIBUTION IS UNLIMITED		
17. DISTRIBUTION STATEMENT (of the abstract entered in Block 20, if different from Report)		
18. SUPPLEMENTARY NOTES		
19. KEY WORDS (Continue on reverse side if necessary and identify by block number) Laser, infrared; Endothelium; Corneal; Trypan blue; Corneal endothelium damage; Tetrazolium salt medium		
20. ABSTRACT (Continue on reverse side if necessary and identify by block number) Specimens of corneal endothelium of adult rhesus monkeys were evaluated for alterations by one of three staining techniques. One cornea from each animal had been exposed to carbon dioxide laser radiation (at 10.6 μ m, 100 msec, 65, 45, or 35 watts/cm ² , spot diameter of 3.2 mm at the 1/e intensity points) and removed surgically two hours after exposure. The other eye of each of the 18 monkeys served as controls (they were not exposed to laser		

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20. Abstract (cont)

radiation). Six specimens, two at each exposure, were stained with trypan blue (TB); nine specimens, three at each exposure, were stained with tetrazolium salt medium (TNBT); and three specimens, one at each exposure, were stained with hematoxylin. Each control cornea was stained with the same as the matching specimen. All specimens were processed for histologic examination by embedding in Epon. The combined use of TB and TNBT staining procedures allows detection of subtle alterations of the corneal endothelium at irradiances as low as 35 watts/cm² of CO₂ laser (10.6 μ m) radiation for a 100 msec exposure. At irradiances from 35 to 65 watts/cm², a progressive increase in the incidence of altered cellular viability and enzyme activity is induced. As these techniques, in combination, are applied, the response of the corneal endothelium to infrared laser radiation and its dose dependence can be described more accurately.

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ABSTRACT

Specimens of corneal endothelium of adult Rhesus monkeys were evaluated for alterations by one of three staining techniques. One cornea from each animal had been exposed to carbon dioxide laser radiation (at 10.6 μm , 100 msec, 65, 45 or 35 watts/cm², spot diameter of 3.2 mm at the 1/e intensity points) and removed surgically two hours after exposure. The other eye of each of the 18 monkeys served as controls (they were not exposed to laser radiation). Six specimens, two at each exposure, were stained with trypan blue (TB); nine specimens, three at each exposure, were stained with tetrazolium salt medium (TNBT); and three specimens, one at each exposure, were stained with hematoxylin. Each control cornea was stained with the same as the matching specimen. All specimens were processed for histologic examination by embedding in Epon. The combined use of TB and TNBT staining procedures allows detection of subtle alterations of the corneal endothelium at irradiances as low as 35 watts/cm² of CO₂ laser (10.6 μm) radiation for a 100 msec exposure. At irradiances from 35 to 65 watts/cm², a progressive increase in the incidence of altered cellular viability and enzyme activity is induced. As these techniques, in combination, are applied, the response of the corneal endothelium to infrared laser radiation and its dose dependence can be described more accurately.

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PREFACE

We thank Edwin S. Beatrice, MD, Colonel, United States Army Medical Corps, who made many helpful suggestions, both in the design of the project and in the interpretation of the results. The work could not have been carried out without his encouragement, cooperation, and expertise. Thanks is also extended to Lottie B. Applewhite for editorial comments and Ann L. Wilkinson for preparation of the manuscript.

P. Shawalak, MD, is currently affiliated with Jefferson Medical College, Philadelphia, Pennsylvania.

This study incorporated the lengthy embedment procedures that were acceptable prior to 1975. Currently, plastic embedment involves infiltration (2-4 hr at room temperature) in the propylene oxide-Epon mixture, and embedment is carried out overnight (~ 14 hr, 70° C). Since these two steps considerably shorten the overall procedure described in Table 1, they are recommended as a substitution for the last three steps (†).

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CORNEAL ENDOTHELIUM AFTER INFRARED LASER EXPOSURE

Evaluation of Techniques to Study Corneal Lesions

--Schuschereba et al

Alterations in both structure (1-4) and enzyme activity (5) in the various layers of the cornea irradiated by the CO₂ laser (10.6 μ m) have been demonstrated. Fluorescein staining² and slit-lamp observations of the epithelium have been made; however, no evaluation of the corneal endothelium has been made to determine if it is affected at doses which damage the epithelium and superficial stroma. In addition, no specialized histopathological techniques have been used to assess the sensitivity of the endothelium to such irradiances. Therefore, by testing for cell viability (6) and enzyme activity (5), we have sought to evaluate several techniques which could possibly reveal whether or not endothelial cell alteration occurs. The combined use of these techniques will aid in defining the dose-response relationship for the endothelial change.

METHODS

Subjects, Exposure, and Surgical Procedure

The corneas of 18 adult Rhesus monkeys were involved in these studies. A surgical level of anesthesia (sodium pentobarbital intravenous 10 mg/kg of body weight) was used for all procedures. Pupils were dilated with 1% tropicamide (Mydracyl) and 1% cyclopentolate hydrochloride (Cyclogyl).

One cornea of each adult Rhesus monkey was exposed to CO₂ laser radiation at 10.6 μ m. The other cornea in each of the monkeys was used as its control. Irradiances of 65, 45, and 35 watts/cm² were used in this investigation. All exposures had a pulse duration of 100 msec. The intensity distribution was Gaussian with an irradiance diameter of 3.2 mm at the 1/e intensity points. The intensity distribution and irradiance diameter were measured at the exposure plane with a scanning thermocouple. Laser power measurements were made with a calibrated detector (Coherent Radiation Laboratories, Model 201).

After slit-lamp examination of fluorescein-stained corneas, whole eyes were enucleated, within two hours after laser exposure. Each was placed in a dissecting vial lined with saline moistened gauze pads. The corneas, including a 1 mm scleral rim, were excised as atraumatically as possible and rinsed in normal saline. The excision consisted of making a 2 mm lateral incision along the limboscleral junction with a sharp blade and subsequent removal with corneal scissors. After the enucleation, the animals were sacrificed.

Techniques

The exposed corneas were stained by three techniques: trypan blue (TB), 6 specimens; 2,2',5,5'-tetra-p-nitro-phenyl-3,3'-(3,3'-dimethoxy-4,4'-biphenylene) dietetrazolium chloride (TNBT), 9 specimens; and Delafield's hematoxylin, 3 specimens. Each control (unexposed) cornea was stained by the same technique as the paired irradiated specimen.

Trypan blue (Tb). Six corneas, two at each of the three exposure levels, were used. After a brief rinse, excess saline was blotted from the corneal cup with bibulous paper. Two drops of 0.5% trypan blue in normal saline solution were placed directly into the inverted corneal cup, contacting only the endothelium. After two minutes of staining, the corneas were rinsed in saline and the endothelium immediately photographed. Specimens were then transferred to 0.1 M phosphate buffered 3.0% glutaraldehyde solution and embedded in plastic (Table 1).

Tetrazolium salt (TNBT). A total of nine corneas, three from each exposure level, were evaluated after excision, corneas were incubated at 37 C for six hours in TNBT medium (Table 2) for demonstration of di- and triphosphopyridine nucleotide (DNPH and TPNH) activity (7). After incubation, the corneas were fixed in 10% 0.1 M phosphate buffered formalin for one week. One specimen from each exposure was hydrated (normal saline) and topically stained with hematoxylin. All tissues were then processed by standard plastic embedding methods (Table 1) and sectioned at 3-4 microns for histologic observation.

Hematoxylin. A total of three corneas, one from each exposure level, was used. Immediately after excision corneas were topically stained for 1 minute with aqueous hematoxylin (1:1) as a baseline evaluation against which the TB and TNBT results could be compared. After initial observation, corneas were fixed in 0.1 M phosphate buffered (pH 7.3) 3.0% glutaraldehyde, embedded in plastic (Table 1) sectioned at 3-4 microns, and photographed.

RESULTS AND DISCUSSION

Gross Observations

At 65 watts/cm² (Figure 1a), 45 and 35 watts cm/² (Figure 1b) the corneas were opacified.

Table 1. Cytochemical and Histological Techniques

Staining	^a ₁ Stain trypan blue (0.5%)	2.0 min
	or	
	^b ₁ Incubate TNBT	6 hr 37C
Fixation	^a ₂ 3.0% glutaraldehyde in 0.1 M phosphate buffer (pH 7.3)	4 hr
	or	
	^b ₂ 10% formalin in 0.1 M phosphate buffer (pH 7.3)	1 wk
Wash	0.1 M phosphate buffer (pH 7.3)	30 min
Staining	*Delafield's hematoxylin (topically 1:1)	2 min
Dehydration	Graded ethanols	4.5 hr
	Propylene oxide	1.5 hr
Infiltration	Propylene oxide - Epon (stoppered 1:1)	‡4 hr 45C
	Propylene oxide - Epon (unstoppered 1:1)	‡14 hr 37C
Embedding	New 100% Epon	‡3 days 60C

^aTrypan sequence. ^bTNBT sequence. *Optional. †See third paragraph of Preface.

Table 2. Incubation Medium (After Yanoff and Tsou (7))

Tetrazolium salt	10 mg
Reduced diphosphopyridine nucleotide (DPNH)	20 mg
Phosphate buffer 0.1 M (pH 7.2)	10 ml
Sucrose	1.25 g



Figure 1. Slit lamp photographs of the corneas of two rhesus monkeys, one hour after exposure to CO₂ laser irradiation (10.6 μ m) for 100 msec. The corneal irradiances of the lesions were 65 watts/cm² in a (arrow) and 45 watts/cm² (left lesion) and 35 watts/cm² (right lesion) in b (arrows).

Direct observation of the corneal endothelium after excision and TB staining revealed focal areas of lightly stained cells (Figures 2a, b, c, and d). These stained areas appeared as patches of cells in a film-like layer in the corneal cup. Usually this layer of cells floated away in the bathing solution before photography could be completed.

The TNBT-staining of the corneal button revealed that only the opacified epithelium did not stain deeply, while the surrounding cornea and controls turned brownish-purple. The staining difference could not be detected on the endothelial side sufficiently because of lack of contrast.

Hematoxylin staining resulted in an overall blue-darkening of the cornea which also precluded endothelial observation. The stain uptake, however, was reduced at the opacified site.

Light Microscopy

Trypan blue (TB). The TB technique at 65 watts/cm² showed marked endothelial cell staining (Figures 2a and 2b) beneath the center of the lesion (approximate size of stained area 1.5 mm in diameter). Some peripheral TB staining surrounded the lesion but in an incomplete circumferential pattern (Figure 2c). At the lower irradiances directly beneath the exposed site, only a few endothelial cells absorbed stain which gave the appearance of diffuse localization (Figure 2d). The number of stained cells decreased as the irradiances were lowered. No peripheral endothelial cell staining was observed at 45 and 35 watt/cm² levels (Figure 2d) and no staining of the cells occurred in the controls (Figure 2e).

Histologic sections of the 65 watt/cm² lesions usually

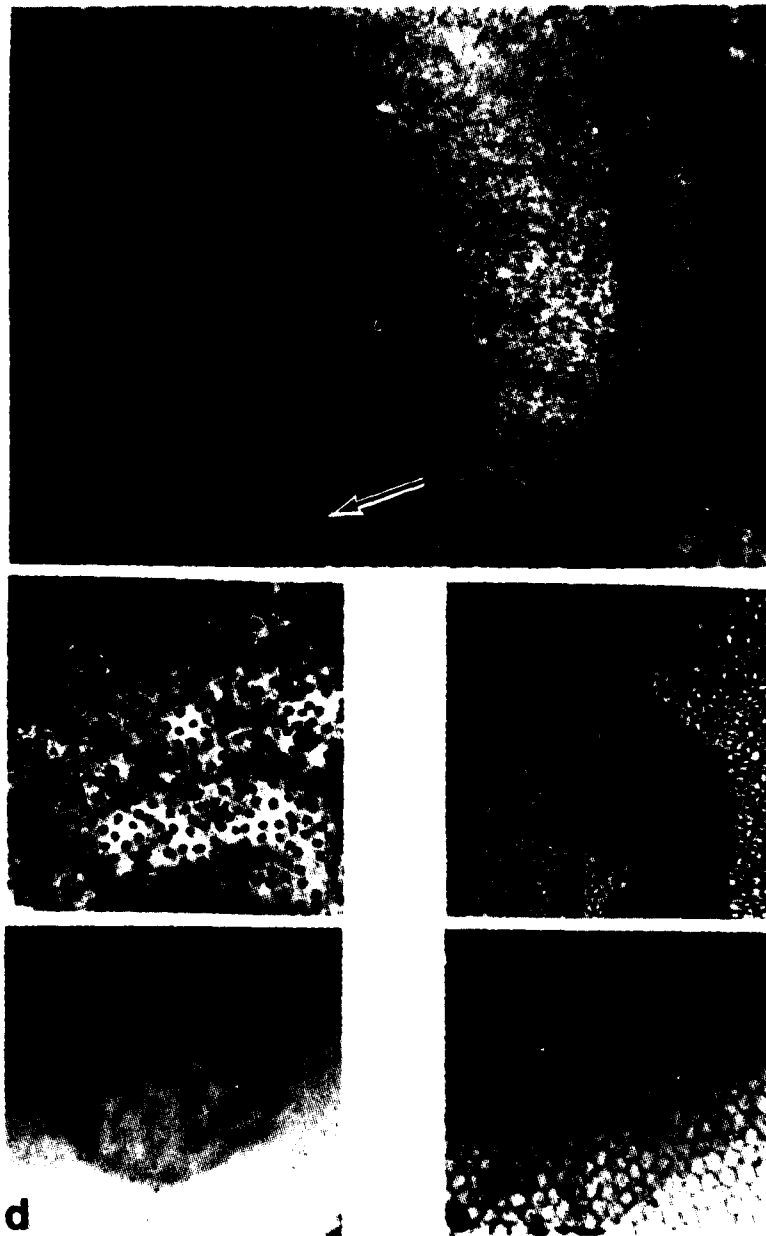


Figure 2. Trypan blue (TB) staining of the cornea. **a.** TB staining of the irradiated (65 watts/cm^2) site two hours post exposure and a view of the epithelial surface (X35). Ridges (*arrow*) of the epithelial surface extend from the center of the lesion to the periphery (P); asterisks (*) indicate areas where the endothelium (beneath this view of the lesion) was stained with TB, as seen in **b** and **c**. **b.** View of the stained underlying endothelial cells (X100) from **a** (left side). **c.** Peripheral island from area beneath the lesion's edge (X100) from **a** (right side). **d.** TB staining of the endothelium two hours post exposure (35 watts/cm^2 , X25); the stained cell clusters (*arrows*) are diffusely localized beneath the center of the lesion. **e.** The control (non-irradiated) endothelium treated with TB (X150) did not take up the stain.

demonstrated missing endothelial cells. At the lower levels of irradiation, the sections had focal areas of lost cells, while the remaining cells appeared normal. On longitudinal sections of the cornea, the number of missing cells decreased with decreasing irradiance. The additional processing of the corneas, after TB staining, probably contributed to the loss of some endothelial cells.

Tetrazolium salt (TNBT). All irradiated corneas incubated in TNBT medium and subsequently sectioned indicated that the DPNH and TPNH activity in the epithelium at the lesion center was decreased as evidenced by a decreased formazan precipitate (Figure 3). Others (5) have found, at the 65 watt/cm² level, an increase in enzyme activity at both the epithelium and endothelium, while we clearly demonstrated that enzyme activity decreased at the epithelium and increased at the endothelium. Our demonstration, however, was at the 45 watt/cm² rather than at the 65 watt/cm² level. The differences in the two findings may lie in the amount of time allowed before and during the corneal incubation (Table 1). In our study we allowed two full hours before incubation, this period of time allowed metabolism to accommodate activation or inactivation, and, therefore, the clinical picture is represented more accurately. In addition, we demonstrated that the basal epithelial cells at the lesion periphery showed a preferential TNBT uptake (Figure 3) similar to that previously reported for the normal dog cornea (7). Topical counterstaining with hematoxylin resulted in the blue staining of the superficial cell layers which could be easily distinguished from the brownish-purple formazan of the TNBT which also gave an outline to the otherwise invisible tissue (Figure 3).

Inadequate specimen preparation, due to endothelium friability, precluded an accurate determination of endothelial cell enzyme activity in the 65 watt/cm² lesions. We were, however, able to demonstrate a denser formazan precipitate in the endothelium of the 45 watt/cm² lesions, but not of the 35 watt/cm² lesions. TNBT staining, therefore, suggests an elevated endothelium cell metabolism for the 45 watt/cm² exposures. The precipitate at the 35 watt/cm² level could not be distinguished from controls. This could be due to one of three reasons or their combinations: 1) The endothelial metabolism was not altered at this dose, 2) the precipitate density was too subtle for the human eye to detect a density difference when compared to controls, 3) the saline rinse neutralized any metabolic differences due to an osmotic or temperature effect.

Hematoxylin. Corneas stained with hematoxylin demonstrated stain uptake where cell junctions were broken (Figure 4a) and a selective reduced staining of the epithelium at opacified sites (Figures 4b and c). Where cells were lost prior to staining, a stain penetration of up to 100 μ m occurred into the stroma (Figure 4c). The lower irradiances (45 and 35 watts/cm²) produced no discernible changes as evidenced by the topical staining with hematoxylin.

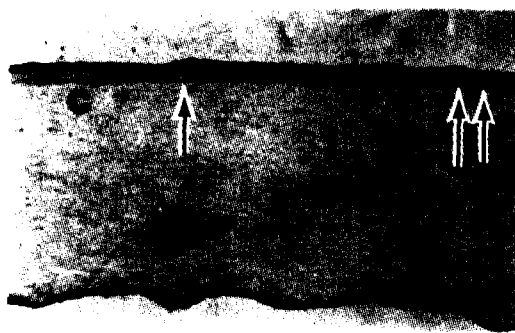


Figure 3. Lesion stained with tetrazolium salt medium (TNBT) and counterstained with hematoxylin (45 watts/cm², X450). Note that the enzyme activity is suppressed near the center of the lesion (c) and enhanced at the periphery (p). Basal epithelial cells show less stain uptake at the center (single arrow) than at the edge (double arrow).

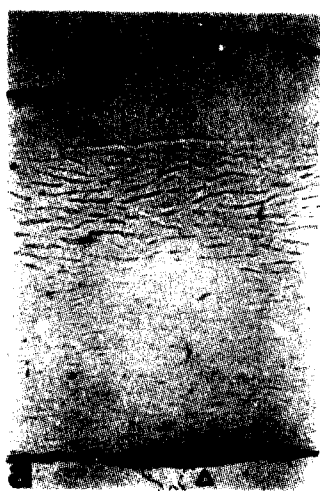


Figure 4. Hematoxylin staining two hours after irradiation at 65 watts/cm². **a.** A large vacuole (v) is present in the epithelium at the periphery of the lesion (X450); note the dense staining and absence of endothelium beneath the vacuole (Δ). **b.** Photograph (X1000) illustrates the epithelial undulation and loss of cellular definition in the lesion center; the undulation represents, in cross section, the ridges illustrated in Figure 2a.



c. In the lesion center, the descement membrane and the stromal keratocytes show stain uptake where the endothelial cells were lost (X450); note the undulation of the epithelium and depth of staining; compare with the staining depth at the periphery in Figure 3.

CONCLUSIONS AND RECOMMENDATIONS

The combined use of TB and TNBT staining procedures allows detection of subtle alterations to the corneal endothelium at irradiances as low as 35 watts/cm² of CO₂ laser (10.6 μ m) radiation for a 100 msec exposure. Based on the incidence of endothelial cell staining with TB, few (if any) endothelial alterations would be expected at irradiances below 35 watts/cm² for 100 msec exposures. At the light microscope level and with the use of the TNBT technique the 35 watts/cm² irradiance does not appear to alter endothelial cell enzyme activity. At irradiances from 35 to 65 watts/cm² a progressive increase in the incidence of altered cellular viability and enzyme activity is induced. As these techniques, in combination, are applied, the response of the corneal endothelium to infrared laser radiation and its dose dependence can be described more accurately.

Quantitative analysis of precipitate density at the electron microscope level should provide a means for improved assessment of DPNH and TPNH activity. The quantitative approach is feasible because TNBT formazans can be complexed with osmium tetroxide to form electron opaque deposits (7).

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